Long non-coding RNA Linc00312 modulates the sensitivity of ovarian cancer to cisplatin via the Bcl-2/Caspase-3 signaling pathway

Chuanqi Zhang, Min Wang*, Cong Shi, Fanli Shi, Cheng Pei

Department of Gynecology and Obstetrics, Shengjing Hospital Affiliated of China Medical University, Shenyang, Liaoning, China;

Summary
Chemotherapy is one of the main treatments for ovarian cancer (OC). Cisplatin combined with paclitaxel is a commonly used chemotherapy regimen. However, effective cancer therapy is hindered by a patient’s resistance to cisplatin. The mechanism that potentially leads to that resistance is unclear. The current study examined the mechanism by which Linc00312 is involved in resistance to cisplatin in OC. Quantitative real-time PCR (RT-qPCR) was used to test for expression of Linc00312 in freshly frozen tissue samples of OC and in SKOV3 and SKOV3/DDP cells. In situ hybridization was performed to examine the distribution of Linc00312 expression in paraffin-embedded histological sections that were sensitive or resistant to cisplatin. The cell counting kit-8 assay was used to detect cell viability. Flow cytometry was used to measure cell apoptosis. RT-qPCR was performed to confirm changes in expression of MDR1, MRP1, Bcl-2, Bax, Caspase-3, and Caspase-9 mRNA. Levels of MDR1, Bcl-2, Bax, Caspase-3, and Caspase-9 protein were detected with Western blotting. Experiments indicated that the expression of Linc00312 decreased significantly in SKOV3/DDP cells compared to that in SKOV3 cells. Upregulation of Linc00312 can considerably increase the sensitivity of SKOV3/DDP cells to cisplatin, while down-regulation of Linc00312 has the exact opposite effect in SKOV3 cells. Linc00312 enhanced the sensitivity of SKOV3/DDP cells to cisplatin by promoting cell apoptosis via the Bcl-2/Caspase-3 signaling pathway. These findings suggest that Linc00312 may be a promising clinical strategy for the treatment of drug-resistant OC.

Keywords: Linc00312, cisplatin, chemosensitivity, ovarian cancer, cell apoptosis

1. Introduction

Ovarian cancer (OC) is one of the most common threats to women's health worldwide; OC has a high incidence and mortality, so molecular biomarkers for OC need to be identified and the pathogenesis of OC needs to be determined in order to devise an effective therapy (1). Numerous studies have established that long non-coding RNAs (LncRNAs) are widespread and potent regulators of diseases, so LncRNAs could serve as biomarkers and targets in OC.

LncRNAs, a class of RNA longer than 200 nucleotides that cannot encode proteins, are mRNA-like transcripts that have been increasingly identified as the key regulators in the tumorigenesis and progression of various human cancers (2). Thus, investigating the roles and potential mechanisms of LncRNAs in tumorigenic driver pathways is of considerable interest (3). One such LncRNA is Linc00312. Linc00312 is transcribed from the genomic region of chromosome 3p25.3, and Linc00312 has been found to be significantly dysregulated in a wide range of diseases, such as non-small cell lung cancer (4), bladder cancer (5), thyroid cancer (6) and nasopharyngeal carcinoma (7). Furthermore, Linc00312 plays a vital role in cell proliferation (8), cell apoptosis, differentiation, and metastasis (7) by interacting with particular microRNAs.

*Address correspondence to:
Dr. Min Wang, Department of Gynecology and Obstetrics, Shengjing Hospital Affiliated of China Medical University, No. 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning, China.
E-mail: wm21st@126.com

DOI: 10.5582/bst.2018.01052

Released online in J-STAGE as advance publication June 28, 2018.

www.biosciencetrends.com
or target genes.

However, there is no evidence to prove whether Linc00312 is connected to drug resistance, especially in OC. This study is the first to describe the functions of Linc00312 in the sensitivity of OC to cisplatin.

2. Materials and Methods

2.1. Patient samples

Freshly frozen tissue samples of serous epithelial ovarian carcinoma (EOC) from chemosensitive patients \((n = 60)\) and chemoresistant patients \((n = 60)\) were retrospectively collected at Liaoning Cancer Hospital and Institute from 2014 to 2016. Paraffin-embedded histological sections came from 15 patients in each group from 2013 to 2015. All patients underwent tumor excision, followed by 6-8 cycles of chemotherapy with cisplatin and paclitaxel. In accordance with NCCN guidelines, the chemoresistant group responded to initial chemotherapy but failed to respond during later chemotherapy or within 6 months of the conclusion of chemotherapy. The chemosensitive group were patients with recurrence after 12 months or with no recurrence after chemotherapy. Clinical information including age, stage of surgery, tumor grade, pathological subtype, and lymph node metastasis was obtained from medical records. This study was approved by the Ethics Committee of China Medical University's Shengjing Hospital.

2.2. Cell lines and culture

The human ovarian cancer cell line SKOV3 were purchased from the Cell Culture Collection of Shanghai. Cisplatin-resistant SKOV3/DDP cells were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Australian FBS; Cellmax Cell Technology Co., Ltd, Lanzhou, China) and 1% penicillin/streptomycin (HyClone) in a humidified incubator at 37°C in an atmosphere of 5% CO₂. To maintain resistance, SKOV3/DDP cells were cultured in the presence of 1 μg/mL cisplatin (Meilune Biotechnology Co., Ltd., Dalian, China). Cells in the logarithmic phase of growth were used in all experiments.

2.3. Cell transfection

An Linc00312-overexpression plasmid and a control plasmid were purchased from Genechem Co., Ltd. (Shanghai, China). SiRNAs (GenePharma Co., Ltd., Shanghai, China) for Linc00312 and negative controls (NC) were synthesized. SKOV3 and SKOV3/DDP cells were counted and seeded in 6-well plates with antibiotic-free medium for 24 h to ensure 70% cell confluence on the day of transfection. Cisplatin was transfected with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA isolation was performed 48 h after transfection, and protein extraction was performed 72 h after transfection. SiRNA was transfected 3 times at a final concentration of 30 nM.

2.4. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from tumor cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 400ng of total RNA from each sample was converted to cDNA using the PrimeScriptVR RT Reagent Kit (Takara, Dalian, China). RT-qPCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China) on the Stratagene Mx3000P Real-Time PCR System (Agilent Technologies, USA) according to the manufacturer's instructions. The primers for analysis are listed in Table 1. Gapdh mRNA served as an endogenous control, and relative levels of expression of other mRNAs were measured using the 2−ΔΔct method.

2.5. Cell counting kit-8 assay (CCK-8 assay)

SKOV3 cells and SKOV3/DDP cells were plated on 96-well plates at a density of 8×10³ cells/100 μL of medium per well. After transfection for 24 h, cisplatin was added to each well at a concentration of 0, 1, 2, 4, 8, 12, 16, 24, 32, or 60 μg/mL. Cells were incubated for 48 h. Cell viability was assessed using the CCK-8 assay (Dojindo Laboratories, Shanghai, China). After 1 h, the

---

Table 1. The primers for RT-qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence ( from 5’ to 3’), Sense</th>
<th>Sequence ( from 5’ to 3’), Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linc00312</td>
<td>TCTGGCTGTGTTGTGTTGTTGGA</td>
<td>GCCTATTGCTTTGGTTTCTGGCT</td>
</tr>
<tr>
<td>MDR1</td>
<td>GACCGGACATCCATCCATGCTT</td>
<td>TGTCCTGGAACCCATGGAAC</td>
</tr>
<tr>
<td>MRP1</td>
<td>TCTACCTCTGTGGCTGAACTGT</td>
<td>CCGATTGTCCTTGCTCTCTCATG</td>
</tr>
<tr>
<td>Bel-2</td>
<td>AAGACGACGAGAGTGGAAAAAGG</td>
<td>GGCGAAAGAAATGCAATGGAATG</td>
</tr>
<tr>
<td>Bax</td>
<td>CTGACGGAGTGTCTCAGAGCCG</td>
<td>CCACCGTAGGATGGCCGTC</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>GGATCCTGTTAGAAGTCTAA</td>
<td>CGGCTCTCACGTGTTATT</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>CCAAGGUACAGGTGACACCT</td>
<td>CTCCATGCCTAGGATGGAAT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CAGGACGTGTGTTGATGAT</td>
<td>GAAAGCTGGGOGCTCATT</td>
</tr>
</tbody>
</table>

---

www.biosciencetrends.com
Downregulation of Linc00312 in chemo-resistant Ovarian Cancer


absorbance of each well was measured at a wavelength of 450 nm using a spectrophotometer (XFLUOR4 Version: V 4.51). The 50% inhibitory concentration (IC_{50}) value for cisplatin treatment was estimated based on the viability curve.

2.6. Cell apoptosis assay

Cells were plated at a density of 2 × 10^3 cells/2 mL medium on 6-well plates for 24 h. After transfection for 24 h, cells were treated with 3 μg/mL cisplatin. After 48 h of treatment, cells were harvested and washed twice with cold PBS. Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (Yuheng Co., Ltd, Jiangsu, China). Cells were stained with 5 μL Annexin V and 2 μL PI in the binding buffer for 15 min in the dark and then subjected to flow cytometry (BD FACSDiva TM Fusion).

2.7. Western blot analysis

Whole-cell lysates were prepared and quantified and then protein was separated on 10% SDS-PAGE gel. Protein was harvested using RIPA lysis buffer (Beyotime, China), diluted with loading buffer containing SDS (Beyotime, China), and denatured at 100°C for 5 min. After electrophoresis, protein (30 μg) was transferred to PVDF membranes (Millipore, Boston, MA, USA) and then blocked with 5% BSA. The membranes were incubated overnight with primary antibodies from Wanleibio (Shenyang, China) against MDR1 (1:500, Rabbit), Bcl-2 (1:500, Rabbit), Bax (1:500, Rabbit), Caspase-3 (1:1,000, Rabbit), Caspase-9 (1:1,000, Rabbit), and Gapdh (1:1,000, Rabbit), Bax (1:500, Rabbit), Caspase-3 (1:1,000, Rabbit), and Gapdh (1:1,000, Rabbit) as a visual loading control. After membranes were incubated with the secondary antibody (1:4,000, Mouse) as a visual loading control. After membranes were incubated with the secondary antibody (1:4,000, Mouse) and then incubated with a blocking solution, biotinylated anti-mouse digoxin, SABC, and biotinylated peroxidase at 42°C, stained with DAB, and then counterstained with hematoxylin. Finally, tissues sections were dehydrated and mounted.

Two pathologists examined all of the sections and graded them independently. Five views were randomly selected at 400× magnification. A positive hybridization signal was identified by dark brown staining in the cytoplasm. Depending on the intensity of positive staining, staining was scored as follows: negative = 0, weak = 1, moderate = 2, and strong = 3. The proportion of stained cells was scored as < 10% = 1, 10-50% = 2, > 50% = 3. The final score was the product of the two aforementioned scores: 0:(−), 1-3: (+), 4-6: (++), and 7-9: (+++).

2.9. Statistical analysis

SPSS software Version 17.0 (IBM SPSS, Chicago, IL) was used for statistical analysis. The Student's two-tailed t-test was used to evaluate the statistical relevance between different groups. All data are expressed as the mean ± the standard deviation (SD). A value of p < 0.05 was considered statistically significant. All experiments were performed in triplicate.

3. Results

3.1. Downregulation of Linc00312 in chemo-resistant serous EOC tissues

In this study, the level of Linc00312 expression decreased significantly in OC chemo-resistant tissues according to RT-qPCR. The expression of Linc00312 in chemo-sensitive tissue samples was normalized as shown in Figure 1A. The level of expression was significantly lower in chemo-resistant tissues (p < 0.01). The localization of Linc00312 in cells was determined using ISH. Results indicated that Linc00312 was mainly localized in the cytoplasm (Figure 2), and the same trend was as was noted in RT-qPCR was evident (Table 2).

Receiver operating characteristics (ROC) curve analysis was performed to evaluate the accuracy with which patients with OC who were resistant to chemotherapy could be distinguished from those who were sensitive to chemotherapy. A cut-off value was obtained. An area under the curve (AUC) of 0.907 had a high discriminatory power (95% CI: 0.854-0.960, p < 0.01) (Figure 1B). When the cut-off point was 0.717, the chemo-resistance of OC was predicted with a sensitivity of 91.7% and a specificity of 80%. These results suggest that decreased expression of Linc00312 in patients with OC could serve as a biomarker with which to determine the chemo-resistant features of serous EOC.
3.2. Linc00312 reduces chemoresistance to cisplatin in SKOV3 and SKOV3/DDP cells

RT-qPCR results revealed that SKOV3/DDP cells had a lower level of Linc00312 expression than that in SKOV3 cells ($p < 0.01$, Figure 3A).

![Figure 1. Linc00312 expression and ROC curve analysis in chemo-sensitive tissues and chemo-resistant tissues of freshly frozen tissue samples in serous epithelial ovarian carcinoma. (A) Levels of expression according to RT-qPCR. **$p < 0.01$ vs. chemo-sensitive tissues. (B) ROC curve for the value.](image1)

![Figure 2. Expression of Linc00312 in paraffin-embedded histological sections of serous epithelial ovarian cancer by ISH. (A,C) chemosensitive tissue, A×200, C×400; (B,D) chemoresistant tissue, B×200, D×400.](image2)

![Figure 3. Expression of Linc00312 in cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines according to RT-RT-QPCR, normalized to Gapdh. (A) Level of Linc00312 mRNA in SKOV3 and SKOV3/DDP cells. **$p < 0.01$ vs. SKOV3 cell group. (B) The level of Linc00312 mRNA transfected with Linc00312 siRNA in SKOV3 cells, *$p < 0.05$ and **$p < 0.01$ vs. si-NC group. (C) Expression of Linc00312 in SKOV3/DDP cells transfected with an overexpression plasmid, *$p < 0.05$ vs. SKOV3/DDP NC group.](image3)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Linc00312 expression</th>
<th>High cases (N)</th>
<th>High cases Rates (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>15</td>
<td>- 0 8 6 1</td>
<td>7 46.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance</td>
<td>15</td>
<td>0 11 4 0</td>
<td>4 26.7</td>
<td></td>
<td>0.0437*</td>
</tr>
</tbody>
</table>

*Sensitivity vs. resistance, *$P = 0.0437$
To further investigate the effect of Linc00312 on cell proliferation and cisplatin resistance, siRNA was transfected into SKOV3 cells and GV146-Linc00312 into SKOV3/DDP cells (Figures 3B and C). Cells were treated with different concentrations of cisplatin. Inhibition rates and IC\textsubscript{50} values were determined with a CCK-8 assay. After treatment with cisplatin, the rate of inhibition of SKOV3-siRNA cells decreased significantly compared to that in SKOV3-NC cells (p < 0.05, Figure 4A). The rate of inhibition of SKOV3/DDP cells with GV146-Linc00312 increased markedly (p < 0.05, Figure 4B). In SKOV3 cells transfected with siRNA, the IC\textsubscript{50} of cisplatin was 6.308 ± 0.299 µg/mL, which was higher than 4.297 ± 0.148 µg/mL in control SKOV3 cells. The IC\textsubscript{50} of cisplatin decreased to 4.609 ± 0.551 µg/mL in SKOV3/DDP cells transfected with GV146-Linc00312 in comparison to that in control SKOV3/DDP cells (8.817 ± 0.988 µg/mL) (p < 0.01, Figure 4C). These results indicate that Linc00312 acted as a key factor for the chemoresistance of SKOV3/DDP cells to cisplatin.

3.3. Linc00312 suppresses chemoresistance to cisplatin through activation of cell apoptosis

Resistance to apoptosis is one of the key reasons for a poor response to cisplatin, so the current authors hypothesized that Linc00312 might be involved in cisplatin resistance through activation of cell apoptosis. After transfection, cells were exposed to 3 µg/mL cisplatin for 48 h. Flow cytometry analysis indicated that suppressing Linc00312 expression in SKOV3 cells decreased cell apoptosis in an inverse manner (17.4000 ± 0.55678 vs 21.0667 ± 1.65630, p < 0.05, Figures 5A and B, Figure 6) and that overexpression of Linc00312 in SKOV3/DDP cells increased cell apoptosis (10.3667 ± 0.92916 vs 6.6333 ± 0.45092, p < 0.01, Figures 5C and D, Figure 6).

3.4. Linc00312 activates the Bcl-2/Caspase-3 apoptotic signaling pathway

As mentioned earlier, the overexpression of Linc00312 promoted sensitivity to cisplatin and increased the rate of inhibition of SKOV3/DDP cells, resulting in
significantly increased cell apoptosis. To explore the signaling pathway for this response, the mRNA of drug resistance-related genes (MDP1 and MRP1) and apoptosis genes (Bcl-2, Bax, Caspase-3, and Caspase-9) was detected with RT-qPCR after transfecting cells with Linc00312 siRNA and an overexpression plasmid. When SKOV3 cells were transfected with siRNA, the expression of MDR1, MRP1, and Bcl-2 mRNA increased while the expression of Bax, Caspase-3, and Caspase-9 mRNA was lower than that in SKOV3 NC cells ($p < 0.05$, Figure 7A). When GV146-Linc00312 was transfected into SKOV3/DDP cells, the reverse trend was evident ($p < 0.05$, Figure 7B). Western blotting was used to detect levels of MDR1, Bcl-2, Bax, Caspase-3, and Caspase-9 protein, and results revealed

Figure 6. Column chart of the ratio of apoptosis in SKOV3 and SKOV3/DDP cells. *$p < 0.05$ vs. si-NC group and **$p < 0.01$ vs. GV146-NC group.

Figure 7. Levels of mRNA expression of several genes in (A) SKOV3 cells, *$p < 0.05$ vs. si-NC group, and (B) SKOV3/DDP cells, *$p < 0.05$ vs. GV146-NC group.

Figure 8. Levels of expression of MDR1, Bcl-2, Bax, Caspase-3, and Caspase-9 protein in SKOV3 and SKOV3/DDP cells. (A) Respective Western blots of protein expression in SKOV3 cells transfected with Linc00312 siRNA or SKOV3/DDP cells transfected with a GV146 plasmid. The column chart shows quantified expression in SKOV3 cells (B) and SKOV3/DDP cells (C). *$p < 0.05$ vs. si-NC group or GV146-NC group.
the same trends as were noted in levels of mRNA (p < 0.05, Figure 8).

4. Discussion

OC is frequently treated with chemotherapy with cisplatin and paclitaxel, and primary chemotherapy is successful for 80-90% of patients (9). However, patients eventually become resistant to cisplatin, so chemoresistance significantly hampers chemotherapy for OC and patient prognosis (10).

According to recent studies, Linc00312 plays a key role as a tumor suppressor gene. It is down-regulated in several cancer tissues compared to normal tissues, including non-small cell lung cancer (4), bladder cancer (5), thyroid cancer (6), and nasopharyngeal carcinoma (7). At present, however, the expression of Linc00312 and its possible role in chemoresistance in OC has not been studied. The current study found that the expression of Linc00312 was much lower in 60 samples of serous EOC chemo-resistant tissues than that in 60 samples of chemo-sensitive tissues according to RT-qPCR. Furthermore, Linc00312 was mainly localized in the cytoplasm of serous EOC epithelial cells, and its expression according to ISH followed the same trend as that according to RT-qPCR. The ROC curve implied that Linc00312 could be a potential marker with which to distinguish chemo-resistant serous EOC from chemo-sensitive serous EOC.

SKOV3 and SKOV3/DDP cell lines were used to examine Linc00312 expression in cisplatin-resistant OC via in vitro experiments. The restoration of Linc00312 sensitized SKOV3/DDP cells to cisplatin and enhanced cisplatin-mediated apoptosis. Inversely, restraining Linc00312 decreased the responsiveness of SKOV3 cells to chemotherapy. This indicated that Linc00312 may be a promising marker for the identification of chemotherapy-resistant and sensitive OC and a biomarker for use in patients who have been diagnosed with OC and who are receiving platinum-based chemotherapy.

To ascertain the possible mechanism for this phenomenon, siRNA was transfected into SKOV3 cells and an overexpressed plasmid was transfected into SKOV3/DDP cells. Knockdown of Linc00312 promoted the resistance of OC cells to cisplatin by suppressing cell proliferation and inducing apoptosis, whereas overexpression of Linc00312 sensitized OC cells to cisplatin.

Multi-drug resistance (MDR) is mainly due to ATP-binding cassette transporters, and this phenomenon explains why few patients with cancer respond well to chemotherapy drugs (11). Multidrug resistance 1 (MDR1) and multidrug resistance protein 1 (MRP1) are two well-known transporters (12) that are involved in cisplatin-induced resistance (13). The overexpression of MDR1 and MRP1 inhibit intracellular drug accumulation and catalyze energy-dependent drug efflux in different malignancies (14,15), such as OC (16).

Most chemotherapies kill cancer cells mainly by promoting cell apoptosis, and fewer cells are apoptotic when they are chemoresistant. Bcl-2 is mainly located in the mitochondria and rough endoplasmic reticulum, and it participates in the cell apoptosis intrinsic pathway by inhibiting the oligomerization of Bax to prolong the life cycle of cells (17). Bcl-2 overexpression induces the immortalization of damaged cells, it promotes tumor development, and it regulates cell proliferation and apoptosis (18). Bax induces the permeabilization of the outer mitochondrial membrane and it then activates the caspase family to form an apoptotic signaling pathway (19,20). The current results indicated that underexpression of Linc00312 increased the expression of Bcl-2 and decreased the expression of Bax, Caspase-3, and Caspase-9, while overexpression of Linc00312 had the opposite effect on expression of the those genes. These results indicate that Linc00312 acted via the bcl-2/caspase-3 pathway in OC cells.

According to previous studies, the most common way in which LncRNA functions is through competing endogenous RNA (ceRNA) during the transcription and post-transcriptional stages. LncRNA absorbs cytoplasmic miRNAs like a sponge and reduces their abundance, keeping miRNAs from repressing target proteins in various cancers (21). This is also an effective regulatory pathway that is correlated with chemotherapy resistance and that has been found in OC (22,23). In addition, Linc00312 has been found to act as a sponge and down-regulate miR-197-3p, thereby inhibiting the invasion and migration of cancer cells (5,6). Therefore, Linc00312 may adsorb another miRNA and thus allow the expression of a target protein to increase. This in turn activates the Bcl-2/Caspase-3 signaling pathway and it promotes cell apoptosis in OC. This point will be studied further in the future.

In summary, this study found that the expression of Linc00312 decreased significantly in chemoresistant serous EOC tissues compared to chemosensitive tissues. Moreover, results indicated that Linc00312 inhibited cell proliferation and promoted cell apoptosis in SKOV3 and SKOV3/DDP cells. Linc00312 decreases Bcl-2 expression and increases Bax expression, thus activating the Bcl-2/Caspase-3 signaling pathway to promote cell apoptosis, and this counteracts chemo-resistance to cisplatin. Thus, Linc00312 may be a potential target for treatment of cisplatin-resistant OC.

Acknowledgement

This work was supported by the Outstanding Scientific Fund of Shengjing Hospital (Grant No. 201705).
References


(Received March 28, 2018; Revised May 24, 2018; Accepted June 10, 2018)